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Title of Invention: Method for producing a medicament improving stability of polypeptide *in vivo* and its application

Abstract:

A method for producing a medicament improving stability of polypeptide *in vivo* and its application. This invention is directed to a method for linking the polypeptide to human serum albumin *in vivo* or *in vitro* on the basis of organic chemistry so as to enhance the stability of polypeptide *in vivo*. Said method comprises modifying *in vitro* with chemical molecules the polypeptide composed of 5-100 amino acids *in vitro*. The modified polypeptide maintains the bioactivity of the original polypeptide and can be linked to human serum albumin *in vivo* or *in vitro* in a covalent mode to elongate the half life of polypeptide *in vivo* and improve the bioavailability. This invention is advantageous in a simple process. The binding of polypeptide to human serum albumin can be fulfilled *in vivo* or *in vitro* in light of need. Moreover, no products of the reaction from modifying the substance other than polypeptide during the binding process are produced.

Claims

1. A method for producing a medicament improving stability of polypeptide *in vivo*, said method comprising first modifying polypeptide *in vitro* with chemical molecules: i.e., dissolving polypeptide in a salt-type buffering solution of 10-200 mmol/Lol/L, then dissolving the chemical molecules for modification use in a solution of 10-200 mmol/Lol/L; adding the dissolved chemical molecules into the polypeptide solution for a continuation of 30 to 60 min of reaction at room temperature or a continuation of 2 to 20 h of reaction at 4 ℃, alternatively, a continuation of 20 to 40 min of reaction at 37 ℃; passing the above reaction mixing solution through a desalting column and eluting it with a salt-type buffering solution; collecting the eluted polypeptide for lyophylization or non-

lyophylization; directly adding *in vitro* the modified polypeptide to the solution containing 5-20 mmol/Lol/LEDTA human serum albumin for a continuation of 30 to 60 min of reaction at room temperature or a continuation of 2 to 20 h of reaction at 4 ℃, alternatively, a continuation of 20 to 40 min of reaction at 37 ℃; adding a compound in a final concentration of 30-200 mmol/Lol/L and containing free sulfhydryl or the compound containing free amino at the end of the reaction.

2. A production method according claim 1, wherein the said polypeptide is composed of 5-100 amino acids.

3. A production method according claim 1, wherein the said polypeptide contains the structure of all or part of enzyme, or enzyme inhibitor, or antigen, or antibody, or hormone, or interferon, or cytokine, or growth factor or differentiation factor etc.

4. A production method according claim 1 or 3, wherein the said polypeptide is the variant of part of the structure of native protein.

5. A production method according claim 1, wherein the said salt-type buffering solution is phosphate buffering solution, HEPES buffering solution, carbonate buffering solution, citrate buffering solution or borate buffering solution.

6. A production method according claim 1, wherein the said solution is phosphate buffering solution, HEPES buffering solution, carbonate buffering solution, borate buffering solution or water for injection, or DMSO or DMF.

7. A production method according claim 1, 5 or 6, wherein the said salt-type buffering solution or solution has a pH of 6-9.

8. A production method according claim 1, wherein the said desalting column is D- Dextran desalting column, KwikSep Dextran desalting column, Excellulose GF-5 desalting column or PD-10 desalting column Pharmacia #17-0851-01.

9. A production method according claim 1, wherein the said chemical molecule modifying polypeptide may be of a NHS maleimide ester class, MBS, Sulfo-MBS, SMPB, Sulfo-SMPB, GMBS, Sulfo-GMBS, EMCS, Sulfo-EMCS, SMCC, Sulfo-SMCC, SIAB, Sulfo-SIAB, BMPA, Sulfo-KMUS or EMCA; it may also be EDC, DMP, DMA, DMS, DSG, DSS or BS3.

10. A production method according claim 1, wherein the modification mode may be amide bond, imido amide bond, or ester bond.

11. A production method according claim 1, wherein non-ionic surfactant, anionic surfactant or modifier may be added to the poorly soluble polypeptide.

12. A production method according claim 1, wherein the said polypeptide modified by chemical molecule is linked *in vitro* to the specific amino acid of human serum albumin through covalent bond.

13. A production method according claim 12, wherein the said specific amino acid is cysteine or lysine, or other amino acids containing free amino.

14. A production method according claim 12, wherein the said covalent bond is a disulfide bond, or imido ester bond.

15. A production method according claim 1, wherein the said compound containing free sulfhydryl or the compound containing free amino is cysteine, mercaptoethanol, dithiothreitol, Tris, glycine, lysine or ethanolamine.

Description

Technical field

This invention relates to a method for linking the polypeptide to human serum albumin *in vivo* or *in vitro* on the basis of organic chemistry so as to enhance the stability of polypeptide *in vivo*.

The polypeptide concerned in this invention can be the active moiety obtained from native or synthetic polypeptides and has the potential therapeutic effect and thus has the important value for being developed as a drug. The therapeutic effects could be direct or indirect, including prevention, vaccine and drug design etc. These polypeptides having bio-activity can be naturally produced from human body, or by recombinant synthesis, or the molecular isomers of these polypeptides, for example, polypeptidase, enzyme inhibitor, antigen, antibody, polypeptide hormone, cytokine, erythropoietin, interferon, interlukin and so on. They can be the complete molecules or only portions or other polypeptide molecules capable of binding to the cell receptors of these polypeptides, such as the polypeptide molecules having the specific bioactivity screened from the Random Peptide Library.

The polypeptide having bioactivity concerned in the present invention can also be non-humanized and can be extracted from animals, bacteria, fungi or plant, or the whole or part of the protein polypeptide that is completely synthesized.

Background art

Many peptides, especially small molecule polypeptide, have the therapeutic activity, but cannot be developed into drugs. There are a number of reasons, including instability *in vivo*, structural instability and difficulty in batch preparation. The failure of many polypeptides in taking effect *in vivo* is caused by the problems like administration dose, administration regimen and pharmacokinetics. The present patent can solve these problems. Polypeptide is linked to human serum albumin without destroying the

structure and physiological functions of polypeptide, yet enhancing the *in vivo* stability so as to elongate the half-life of polypeptide *in vivo* and improving bioavailability.

The present invention is based on the finding as follows: human serum albumin has a longer half-life in serum and can increase the half-life of polypeptide in serum to maintain its activity for a longer period; it can make polypeptide arrive at the action position more effectively so as to reduce the dose of the used polypeptide, improve drug potency and reduce side effects. Besides, small molecule polypeptide has none or little antigenicity and thus is more advantageous than macromolecule protein. It has been reported that the fusion protein from polypeptide and human serum albumin is prepared by gene engineering process so as to increase the half-life of polypeptide *in vivo* (U.S. patent 5,876,969).

Elongating the half-life of drug *in vivo* and improving bioavailability to facilitate clinical administration has always been one of the main drug development directions of pharmaceutical manufacturers and many pharmaceutical institutes. Macromolecule drugs, such as proteins and DNA and RNA-type bio-technique product drugs, are difficult for oral administration because these macromolecules are very easy to be degraded through gastrointestinal tracts; thus these drugs, in most cases, must be administered through subcutaneous, muscle, intravenous or topical injection. However, since any drug only stays in blood for a certain period, macromolecule drugs must be injected repetitively to maintain a certain plasma, which causes a lot of inconvenience to clinical application, especially that many bio-drugs must be taken all one's life. Presently, there are two methods that are in intense study for elongating the half-life of drug *in vivo*: one is changing the composition of reagent, which is generally using polymer to embed drug or to be crosslinked with drug, and the polymer used most is polyethylene glycol, PEG; the other is producing a medical device for controlling the *in vivo* release of drug. PEG-crosslinked bio-drug has been launched into market in US, such as PEG interferon. A lot of PEG-embedding drugs are under clinical tests, such as InfitropinCR of InfiMed Therapeutic (Cambridge, MA), i.e., lasting growth hormone. Besides, many clinical tests for medical devices controlling release are

nearly completed, such as MedPulser ® Electroporation System of Genetronic Biomedical (San Diego, CA). However, due to the incompleteness and complexity of the above two methods, the search of a better method to elongate the half-life of polypeptide drug *in vivo* has a fairly high development value and marketing prospect. A chemical method is used in the present invention to specifically link polypeptide to human serum albumin. The process is simple. The binding of polypeptide to human serum albumin can be achieved *in vivo* or *in vitro* in light of need, and the binding process does not concern the formation of the reactants other than those modifying polypeptides.

As stated above, the polypeptide concerned in the present invention refers to the short polypeptide composed of 5 to 100 amino acids. The active center of most enzymes, or inhibitors of enzyme, or antigens, or antibodies, or hormones, or interferons, or cytokines or growth factors, or differentiation factors are composed of the polypeptides with less than 100 amino acids. Besides, since every protein consists of 20 different amino acids, different combinations of amino acids can constitute any space conformation to fulfill the multiplicity of function. The Polypeptide Random Library obtained by the combinations of random amino acids has the multiplicity much greater than that of the chemical substance library as presently known. The plasticity of polypeptide itself is also better than that of small molecule compounds. Therefore, replacing small molecule compound library with small molecule polypeptide library in some cases also establishes a new route for drug screening.

Identical to protein drugs and small molecule drugs, small molecule polypeptide drugs can also be improved as to its *in vivo* stability and serum half-life by a number of methods, like liposome embedding, sustained release device and linking it to other bio-stable proteins (like antibody Fc fragment or human serum albumin) using recombinant RNA techniques. These methods can solve the problem of *in vivo* stability of most drugs, while having the risk of complex operation or potential immune reaction.

The polypeptide concerned in the present invention can be produced by the expression in *Escherichia coli* using recombinant DNA technique, or by

synthesis using chemical methods. When it is expressed in *Escherichia coli*, the recombinant DNA encoding target polypeptide is cloned into the suitable expression carrier. Said recombinant DNA can be synthesized by classical method, or by the method using polymerase chain reaction (PCR). Introduce the expression plasmid containing target polypeptide recombinant DNA into the suitable host *Escherichia coli*, and obtain the target polypeptide through expression and purification. When polypeptide is synthesized using chemical methods, the automatic solid phase polypeptide synthesizer or standard manual synthesis process can be applied. The known automatic solid phase polypeptide synthesizer can be produced by Applied Biosystems or by other equivalent manufacturers. The standard manual polypeptide synthesis process utilizes the protection provided by *t*-butyloxycarbonyl (*t*-BOC) or 9-fluorenylmethyloxycarbonyl (FMOC) for α-amino of amino acid: the single amino acids are incorporated step by step from C-fragment; then, the synthesized polypeptide is cut from the solid phase attached thereto through deprotection, and the polypeptide is obtained through a series of steps like desalting, extraction and purification.

Small molecule polypeptides have always been widely used in laboratories of immunology. For example, they can be applied to the phenotype analysis of T cell and B cell; they can bind to other immune enhancers or proteins to immunize animals and facilitate the formation of antibody. In recent years, because of the development in Human Genome Project, the understanding of polypeptide has been increasingly enhanced, and the application of small molecule polypeptides also becomes wider. The same as the development of therapeutic monoclonal antibody, the development of small molecule polypeptide drug becomes one of the important research directions of a lot of pharmaceutical manufacturers. For instance, Clinical Test Phase III has been completed at present for Natrecor of SCIOS (Sunnyvale, CA), i.e., B-type natriuretic peptide, BNP, and the final approval from the pharmaceutical authority is being waited for. This drug is the special small molecule polypeptide drug treating acute congestive heart failure. The following table (Table I) lists the size, function and sequence of some representative therapeutic polypeptides.

Table I Some Representative Therapeutic Polypeptides

Name of Polypeptide	Number of amino acid	Sequence (N→C)	Function
B-type natriuretic peptide (BNP)	32	SPKMVQGSGC FGRKMDRISS SSGLGCKVLR RH	Natriuresis, treating acute congestive heart failure
Parathyroid hormone related peptide (PTHrp)	34	CVSEHQLLHD KGKSIQDLRR RFFLHHLIAE IHTA	Treating osteoporosis
EPO mimetic peptide 1 (EMP1)	20	GGTYSCHFGP LTWVCKPQGG	Treating various anemia, the same as EPO
Calcitonin	32	CGNLSTCMLG TYTQDFNKFH TFPQTAIGVG AP	Treating specific types of osteoporosis

Small molecule polypeptides have good therapeutic activity, but they have apparent deficiencies, i.e., instability *in vivo* and unstable structure. Thus, they are very apt to be degraded to produce the problems like administration dose, regimen and pharmacokinetics. In the present invention, polypeptide is linked to human serum albumin *in vivo* or *in vitro* under natural conditions to enhance the *in vivo* stability of polypeptide and improve the therapeutic effects. Such a link is performed under mild conditions or *in vivo*. The linked polypeptide maintains its original functions, and human serum albumin can also be metabolized by the normal pathway.

Disclosure of Invention

The object of the present invention is to provide a method for producing a medicament improving stability of polypeptide *in vivo* and its application.

The object of the present invention is achieved like this: said method

comprising first modifying polypeptide *in vitro* with chemical molecules: i.e., dissolving polypeptide in a salt-type buffering solution of 10-200 mmol/Lol/L, then dissolving the chemical molecules for modification use in a solution of 10-200 mmol/Lol/L; adding the dissolved chemical molecules into the polypeptide solution for a continuation of 30 to 60 min of reaction at room temperature or a continuation of 2 to 20 h of reaction at 4 ℃, alternatively, a continuation of 20 to 40 min of reaction at 37 ℃; passing the above reaction mixed solution through a desalting column and eluting it with a salt-type buffering solution; collecting the eluted polypeptide for lyophylization or non-lyophylization; directly adding *in vitro* the modified polypeptide to the solution containing 5-20 mmol/Lol/LEDTA human serum albumin for a continuation of 30 to 60 min of reaction at room temperature or a continuation of 2 to 20 h of reaction at 4 ℃, alternatively, a continuation of 20 to 40 min of reaction at 37 ℃; adding a compound in a final concentration of 30-200 mmol/Lol/L and containing free sulfhydryl or the compound containing free amino at the end of the reaction.

Dissolve the polypeptide in the phosphate buffering solution of 10-200 mmol/Lol/L, the pH of the buffering solution being 6-9; the buffering solution can be HEPES or carbonate buffering solution, citrate buffering solution or borate buffering solution. For some polypeptides with a poor dissolvability due to the stronger hydrophobicity, some latent solvents like non-ionic surfactants, anionic surfactants or modifiers can be incorporated, such as Tween 20, Tween 60, Tween 80, Triton, sodium dodecyl sulfate, urea or guanidine hydrochloride etc. These examples of buffering solution do not limit the application of other buffering solutions in the present invention by any mode.

Dissolve the chemical molecules for modification use in the phosphate buffering solution of 10-200 mmol/L, the pH of the buffering solution being 6-9; the buffering solution can be HEPES or carbonate buffering solution, citrate buffering solution or borate buffering solution, or water for injection or DMSO or DMF. These examples of buffering solution do not limit the application of other buffering solutions in the present invention by any mode.

The chemical molecule for modification use concerned in the present invention may be of a NHS maleimide ester class, or Sulfo-SMCC, or SIAB, or Sulfo-SIAB, or BMPA, or Sulfo-KMUS or EMCA; it may also be EDC, DMP, DMA, DMS, DSG, DSS or BS3. These examples of chemical molecule do not limit the application of other suitable chemical molecules for modification use in the present invention by any mode to link the target polypeptide to human serum albumin so as to improve the *in vivo* stability of target polypeptide. The molecular symbols and corresponding names and chemical constitution of the above examples of chemical molecules are presented in the following table (Table 2).

Adding the dissolved chemical molecules into the polypeptide solution for a continuation of 30 to 60 min of reaction at room temperature or a continuation of 2 to 20 h of reaction at 4 ℃, alternatively, a continuation of 20 to 40 min of reaction at 37 ℃.

Pass the reaction mixing solution through a desalting column, D-Dextran, and use the phosphate buffering solution of 10-200 mmol/L to elute it. The buffering solution can be HEPES or carbonate buffering solution, citrate buffering solution or borate buffering solution. The pH of the buffering solution is 6-9; the desalting column may be KwikSep Dextran, Excellulose GF-5 or PD-10 column (Pharmacia #17-0851-01). The eluted polypeptide peak that is collected undergoes lyophylization through direct-cooling lyophylizer or cascade lyophylizer. These examples of desalting column do not limit the application of other suitable desalting columns in the present invention by any mode.

The lyophylized polypeptide is dissolved in the water for injection before administration and is applied *in vivo* in the mode of intravenous injection. Or, it can be topically administered by injection according to the specific functions of polypeptides, such as the topical injection administration at tumor position.

The polypeptide peak that is collected from the reaction with the chemical molecule for modification use can also be, without lyophylization, directly

incorporated into the solution of human serum albumin containing 5-20 mmol/L EDTA for a continuation of 30 to 60 min of reaction at room temperature or a continuation of 2 to 20 h of reaction at 4 ℃, alternatively, a continuation of 20 to 40 min of reaction at 37 ℃ to make the modified chemical molecule bind to human serum albumin in the form of disulfide bond or imido ester bond. At the end of the reaction, incorporate the free sulfhydryl-containing compound of a final concentration 30-200 mmol/L like cysteine, sulfhydryl ethanol and dithiothreito, or the free amino-containing compound of 20-200 mmol/L like Tris, glycine, lysine or ethanolamine. The human serum albumin used in the present invention for *in vitro* link can be the commercially available inhomogeneous albumin preparation isolated from human blood, or the homogeneous albumin subtype produced by the recombinant DNA technique.

The reaction mixing solution is sampled in D-Dextran desalting column and eluted by a buffering solution of 10-200 mmol/L. The buffering solution is phosphate buffering solution, HEPES, carbonate buffering solution, borate buffering solution or citrate buffering solution. The pH of the buffering solution is 6-8. The desalting column may also be KwikSep Dextran or Excellulose GF-5. Collect the eluted protein peak. These examples of desalting column do not limit the application of other suitable desalting columns in the present invention by any mode.

Description of Figures

In the following, the invention will be further illustrated by the examples in combination with the figures.

Fig.1 is Sulfo-MBS modification of the calcitonin according to the present invention.
Fig.2 is Sulfo-SMCC modification of the calcitonin according to the present invention.
Fig.3 is SIAB modification of the calcitonin according to the present invention.
Fig.4 is EDC modification of the calcitonin according to the present invention.

Fig.5A is DMA modification of the EPO mimetic peptide 1 according to the present invention.
Fig.5B is the structure of EPO mimetic peptide 1 (EMP1) according to the present invention.
Fig.6 is DSG modification of the EPO mimetic peptide 1 according to the present invention.
Fig.7 is a figure showing a hypoxic-polycythemic mouse bioassay for determining the activity of erythropoietin.
Fig.8 is a peripheral blood reticulocyte cytometry.

Modes of Carrying Out the Invention

In the following, the present invention is further explained by the examples. However, the invention is not limited to the examples. As indicated above, the polypeptide according to the present invention is obtained by a standard recombinant expression or chemical synthesis followed by a purification.

Example 1: NHS maleimide ester modification of parathyroid hormone related peptide (PTHrp)

PTHrp consisting of 34 amino acids was obtained by chemical synthesis. In the last step of chemical synthesis, the amino group protected by *t*-BOC was directly subjected to desalting, extraction by organic solvent, and reversed phase-HPLC purification without deprotection by trifluoroacetic acid (TFA) to obtain PTHrp in high purity. The PTHrp was dissolved in 100 mmol/L phosphate buffer with a pH of 7.0. The final concentration of PTHrp was 100 μM.

NHS maleimide ester Sulfo-MBS was dissolved in 10 mmol/L phosphate buffer with a pH of 7.0. The final concentration of NHS maleimide ester was 2 mmol/L. The solution was formulated according to the manufacturer's instruction one hour prior to use.

The dissolved Sulfo-MBS was added to the PTHrp solution to effect reaction for 30 minutes at room temperature or 2 hours at 4 °C, and stirred carefully to mix homogenously (see Fig.1). MBS is a most commonly used

dual-functional crosslinking agent. At neutral pH, MBS is crosslinked with the sulfhydryl or amino group in a polypeptide. The linking procedure in the present invention was achieved by two separate reactions so as to avoid the linking of the same molecules. The free sulfhydryl groups (SH) were provided by the cysteine residues at the N-terminal of PTHrp. In the first reaction, MBS was linked to PTHrp. In the second reaction (see the examples in the following text), MBS causes a crosslinking between PTHrp and the amino groups of lysine residues in human albumins.

After Sulfo-MBS crosslinking, the PTHrp mixture was subjected to TFA deprotection, and then subjected to desalting and purification by the method in Example 7 to remove the free chemical molecules and the uncrosslinked PTHrp. The efficiency of crosslinking was calculated, and the product was separated and packed for use in laboratory researches such as stability tests, toxicity tests, and efficacy and pharmacokinetic tests.

Example 2: Sulfo-SMCC modification of PTHrp

The PTHrp in high purity was prepared according to the same method in Example 1. The PTHrp was dissolved in 20 mmol/L phosphate buffer with a pH of 7.0. The final concentration of PTHrp was 50 μM.

1 mg Sulfo-SMCC was added to the above solution. The reaction was effected for 60 minutes at room temperature or 30 minutes at 37 °C, and stirred carefully to mix homogenously (see Fig.2). SMCC is a commonly used dual-functional crosslinking agent. Sulfo-SMCC maintains the reactive properties of SMCC, and also has good water-solubility. At neutral pH, Sulfo-SMCC causes crosslinking between protein and polypeptide. The linking procedure in the present invention was achieved by two separate reactions so as to avoid the linking of the same molecules. Firstly, Sulfo-SMCC reacts with the sulfhydryl groups of cysteines in PTHrp. In the first reaction, Sulfo-SMCC was linked to PTHrp. In the second reaction (see Examples 8 and 9), Sulfo-SMCC causes a crosslinking between PTHrp and the amino groups of lysine residues in human albumins.

After Sulfo-SMCC crosslinking, the PTHrp mixture was subjected to

deprotection, and then subjected to desalting and purification by the method in Example 7 to remove the free chemical molecules and the uncrosslinked PTHrp. The efficiency of crosslinking was calculated, and the product was separated and packed for use in laboratory researches such as stability tests, toxicity tests, and efficacy and pharmacokinetic tests.

Example 3: SIAB modification of PTHrp

The PTHrp in high purity was prepared according to the same method in Example 1. The PTHrp was dissolved in 20 mmol/L phosphate buffer with a pH of 7.0. The concentration of PTHrp was 0.1 mg/ml.

1.4 mg SIAB was dissolved in 1 ml DMSO. The solution was formulated according to the manufacturer's instruction one hour prior to use.

10 μl SIAB solution was added to 1 ml PTHrp solution. The reaction was effected for 30-60 minutes at room temperature, and stirred carefully to mix homogenously (see Fig.3). SIAB is a commonly used dual-functional crosslinking agent. At neutral pH, SIAB causes crosslinking between protein and polypeptide. The linking procedure in the present invention was achieved by two separate reactions so as to avoid the linking of the same molecules. Firstly, SIAB reacts with the sulfhydryl groups of cysteines in PTHrp. In the first reaction, SIAB was linked to PTHrp. In the second reaction (see Examples 8 and 9), SIAB causes a crosslinking between PTHrp and the amino groups of lysine residues in human albumins.

After SIAB crosslinking, the PTHrp mixture was subjected to deprotection, and then subjected to desalting and purification by the method in Example 7 to remove the free chemical molecules and the uncrosslinked PTHrp. The efficiency of crosslinking was calculated, and the product was separated and packed for use in laboratory researches such as stability tests, toxicity tests, and efficacy and pharmacokinetic tests.

Example 4: EDC modification of calcitonin

Calcitonin was prepared by a chemical method. The protecting groups at

the amino groups and the lysine residues were not removed. The prepared calcitonin was dissolved in 0.5 ml 100mmol/L MES solution with a pH of 4.5. The concentration of peptide was 1.0 mg/ml.

Within one hour prior to the reaction, 10 mg EDC was dissolved in 1 ml deionized water. 100 μl of the resultant solution was added to the calcitonin solution. The reaction was effected for 120 minutes at room temperature, and stirred carefully to mix homogenously (see Fig.4). EDC is a commonly used dual-functional crosslinking agent. At acidic conditions, EDC causes crosslinking between protein and polypeptide. The linking procedure in the present invention was achieved by two separate reactions so as to avoid the linking of the same molecules. In the first reaction, EDC was linked to the residue at the carboxyl terminal of calcitonin by reaction. In the second reaction (see Examples 8 and 9), EDC causes a crosslinking between calcitonin and the amino groups of lysine residues in human albumins.

After EDC crosslinking, the calcitonin mixture was subjected to deprotection, and then subjected to desalting and purification by the method in Example 7 to remove the free chemical molecules and the uncrosslinked calcitonin. The efficiency of crosslinking was calculated, and the product was separated and packed for use in laboratory researches such as stability tests, toxicity tests, and efficacy and pharmacokinetic tests.

Example 5: DMA modification of the EPO mimetic peptide 1 (EMP1)

EPO mimetic peptide 1 (EMP1) consisting of 20 amino acids was prepared by a chemical synthesis. After desalting, extraction by organic solvent, and reversed phase-HPLC purification, EMP1 in high purity was obtained. By HPLC analysis, the purity >95%. By chromatography, the structure of the product was correct. A disulfide bond is formed between two cysteines of EMP1, such that EMP1 is in a half-ring shape (see Fig.5B). DMA links to the free amino group at N-terminal of EMP1 by reaction (see Fig.5A). The brief reaction is in the following:

5 mg EMP1 was dissolved in 0.5 ml 0.1M phosphate buffer containing 1% DMSO. The buffer had a pH value of 7.4. The solution was sample-loaded

to 10 ml balanced Sephadex G-10 column (Pharmacia# 17-0010-02). The buffer system was still 0.1 M phosphate buffer with a pH of 7.4. Polypeptide peaks were collected, and the EMP1 concentration was adjusted to 2 mg/ml.

DMA solution was formulated into a concentration of 100 mmol/L according to the manufacturer's instruction. EMP1 was slowly dripped into the DMA solution, and mixing homogeneously. The reaction was effected for 60 minutes at room temperature. After reaction, glacial acetic acid in a quarter volume of the sample was added to stop the reaction. Then, the resultant was subjected to desalting and purification by the method in Example 7 to remove the free DMA molecules, the byproduct, and the uncrosslinked EMP1. The efficiency of crosslinking was calculated, and the product was separated and packed for use in stability tests and *in vivo* activity tests.

Example 6: DSG modification of EMP1

The EMP1 peptide was prepared according to Example 5. DSG was prepared according to the manufacturer's instructions. EMP1 was slowly dripped into the DSG solution, and mixing homogeneously such that the molar concentration of DSG is 50 to 100 folds of the molar concentration of EMP1. The reaction was effected for 30 minutes at room temperature or 120 minutes in ice bath (see Fig.6).

20 mmol/L Tris, glycin, lysine, or a solution containing free amino groups was added to stop the reaction. Like Example 5, the resultant was subjected to desalting and purification by the method in Example 7 to remove the free DSG molecules, the byproduct, and the uncrosslinked EMP1. The efficiency of crosslinking was calculated, and the product was separated and packed for use in stability tests and *in vivo* activity tests.

Example 7: Desalting and storage of the modified polypeptide

The crosslinked products in Examples 1, 2, 3, 4, 5 and 6 were sample-loaded to 5 ml D- Dextran desalting column, and was eluted using 100

mmol/L phosphate buffer which had a pH of 7.0. UV detector was used to detect the absorption value of the eluate at 280 nm. The polypeptide peaks were collected, and were diluted with eluate. The product was filled into cillin-glass bottles, 1 ml each, and was freeze-dried for storage. The finally modified polypeptide in the six examples all had a concentration of 1.0 mg/ml.

Example 8: Binding of the modified polypeptide to albumins *in vivo*

The freeze-dried polypeptide was dissolved in 1 ml injection water before administration, and was administered by intravenous injection.

Example 9: Binding of the modified polypeptide to albumins *in vitro*

Determining the molar content of the modified polypeptide peaks in Example 7; adding it to human albumin solution containing 10 mmol/L EDTA; reacting for 30-60 minutes at room temperature, such that the modified molecules bind to human albumins in a form of disulfide bond or imide bond; at the end of reaction, adding a cysteine-containing Tris or glycin buffer having a final concentration of 50 mmol/L, and sample-loading the mixture to 10 ml D- Dextran desalting column; eluting with 10 mmol/L phosphate buffer of 7.0 pH; using UV detector to determine the absorption value of the eluate at 280 nm, and collecting the protein peaks. The desalted polypeptide-albumin peaks could be used for studies of stability tests and *in vivo* activity tests, and could also be further isolated by chromatography so as to thoroughly separate the unbound polypeptide from the polypeptide-albumin crosslinking product and thereby to further enhance the stability *in vivo*. High resolution Q ion exchange chromatography and Superose gel filtration (Pharmacia# 17-0510-01, 17-0536-01) were first choices of the further chromatography.

Example 10: EMP1-albumin crosslinking product still maintained the original erythropoietic activity.

Mouse model is an important method of determining the *in vivo* activity of erythropoietin. In order to increase the sensitivity, a Hypoxic-Polycythemic

Mouse Bioassay was used, wherein the erythropoietic activity *in vivo* of the sample was evaluated by determining the percentage of ^{59}-$FeCl_3$ integrated into hemoglobin. Specific test was in the following: raising laboratory female mice (16-18 g) in a hypoxic bin at 0.6 atmospheric pressure for 3 days; further lowering the pressure to 0.4-0.5 atmospheric pressure and raising mice for 11 days; transferring the mice to normal atmosphere pressure, and conducting tests three days later. ^{59}-$FeCl_3$ purchased from market was diluted with phosphate buffer to 3.7×10exp4 Bq/ml. The mice were randomly divided into 3 major groups, 12 in each major group, and each dose group had 3 mice. By standard EPO administration, the 3 mice were injected with 0.25-1.0 IU/mouse erythropoietin (produced by the applicant), 0.01-0.2 mg/mouse unmodified EMP1, and 0.01-1 μg/mouse EMP1-albumin crosslinking product. Each major group had a control dose group wherein the sample was replaced with normal saline in the same volume (0.2 ml). Two days after the injections, 0.2 ml ^{59}-$FeCl_3$ solution was injected into the abdominal cavity of each animal. The sequence of injection was identical to the sequence of administration. After 48 hours, the animals were Avertin anaesthetic, and were weighed. 0.65 ml blood sample was collected from the aortic arch of each mouse, and the volume of sedimented cells and the amount of isotope were determined. The response of each mouse to drug (i.e., ^{59}-Fe integration percentage in the overall circulating blood) was calculated by the following formula:

cpm(s)×0.075bw(g)×1/cpm(t)×1/v(s)
wherein: cpm(s)=amount of isotope in the sample
cpm(t)=the total amount of injected isotope
bw(g)=body weight (g), the total volume of circulating blood in mouse being 7.5% of body weight
v(s)=sample volume (ml)

A conventional statistic method is used for data treatment. When the volume of sedimented cells was less than 54%, or when a mouse had a body weight of more than 24g, the data would be abandon.

Test results showed that the maximum dose groups of EPO administration (1.0 IU/mouse) were observed to have 20 folds increase ($P<0.0001$) of ^{59}-

Fe integration percentage; at a dose of 0.2 mg/mouse, unmodified EMP1 groups also had a notable increase of ^{59}-Fe integration percentage (about 20 folds, $p<0.0001$); likewise, the EMP1-albumin crosslinking product groups all had increase of ^{59}-Fe integration percentage, and the maximum dose group (1.0 μg/mouse) had as high as more than 40 folds increase of ^{59}-Fe integration percentage (see Fig.7).

Example 11: *in vivo* stability of DMA modified EMP1

There were two simple ways for determining the *in vivo* stability of EMP1. One method is a peripheral blood reticulocyte cytometry. Normal mouse peripheral blood reticulocyte cytometry is an important index for determining the *in vivo* activity of erythropoietin. The *in vivo* stability of polypeptide is indirectly evaluated by the determination of *in vivo* activity. The other method is directly labeling EMP1, determining its half-life in plasma, and thereby directly evaluate its *in vivo* stability. When normal mouse peripheral blood reticulocyte cytometry is used to determine the *in vivo* stability of EMP1, purebred normal mice (16-18g) were randomly divided into 3 major groups, each major group having 7 mouse cages, 6 mice in each cage. The samples were diluted into the following concentrations: 200 IU/ml erythropoietin (produced by Shenyang Sansheng), 10 mg/ml unmodified EMP1, and 1.0 mg/ml modified EMP1-DMA. In the test, 0.2 ml the above samples were injected into each mouse. Each major group had a cage as control group into which 0.2 ml normal saline was injected. At the same day of injection, the control cage was subjected to peripheral blood reticulocyte cytometry. Later, on the 2nd, 4th, 6th, 8th, 10th and 12th days, a cage from each major group was subjected to peripheral blood reticulocyte cytometry. Results showed that EPO groups (40 IU/mouse) were observed to have about 20% increase of peripheral blood reticulocyte cytometry ($P<0.0001$), and the cytometry reached the maximum at the 2nd to 4th days and recovered to normal level after one week; unmodified EMP1 groups also had notable increase of peripheral blood reticulocyte cytometry (about 5%, $P<0.001$), and the cytometry reached the maximum at the 2nd day and recovered to normal level after 3-4 days; likewise, modified EMP1-DMA groups had as high as 110% increase of peripheral blood reticulocyte cytometry, and the cytometry reached the

maximum at the 2nd to 4th days and recovered to normal level after 10 days (see Fig.8). It is deduced that DMA-modified EMP1 has a high *in vivo* stability. Although unmodified EMP1 has surprising activity *in vivo*, the activity does not last long. Maybe, the reason is that, as already known, the excretion system via liver, kidney and tissues rapidly clears away polypeptides.

As indicated above, the half-life of modified EMP1-DMA in plasma can be determined by directly labeling EMP1. Thereby, the *in vivo* stability can be evaluated. Preliminary tests have shown that modified EMP1-DMA has much greater half-life *in vivo* than unmodified EMP1 (a half-life of about 8 hours). Further experiments have to be made to obtain the exact half-life of EMP1-DMA.